JC07 Rec'd PCT/PTO 25 JAN 2002

FORM PTO-1390 (REV. 12-2001) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 1544.03

U S APPLICATION NO. (If known, see 37 CFR 1 5): 10/048193

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR00/00810	July 26, 2000	July 27, 1999

TITLE OF INVENTION: A Human Lactoferrin Produced by Using an Insect Cell and Method ..

APPLICANT(S) FOR DO/EO/US: Hyune Hwan LEE, Yun-Hee CHANG, and Chang-Hee KWEON

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [X] A substitute specification. (the amendment under PCT Article 34 is incorporated)
16. [X] A change of power of attorney and/or address letter. (new application/ new attorney)
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: A substitute drawing
    A copy of the International Search Report
    A copy of the International Preliminary Examination Report

U.S. APPLICATION NO (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/048193	PCT/KR00/00810	1544.03

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1040.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$
Total claims	6 - 20 =	0	x **$18.00**	$
Independent claims	4 - 3 =	1	x **$84.00**	$ 84.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$

TOTAL OF ABOVE CALCULATIONS =	$ 1124.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +	$ 562.00	
SUBTOTAL =	$ 562.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	
TOTAL NATIONAL FEE =	$ 562.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$ 562.00	
	Amount to be refunded:	$
	charged:	$

a. ☒ A check in the amount of $ 562.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0310. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

SIGNATURE

John K. Park
NAME

37,904
REGISTRATION NUMBER

A HUMAN *LACTOFERRIN* PRODUCED BY USING AN INSECT CELL AND METHOD USING THE SAME

TECHNICAL FIELD

The present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same. More particularly, the present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same, in which the method includes the steps of transducing a human *lactoferrin* gene into an insect cell by gene recombination, cloning and expressing human *lactoferrin* in the insect cell, and producing the human *lactoferrin* by using the insect cell.

Human *lactoferrin* is a member of the transferrin family of iron-binding monomeric glycoproteins and also called "*lactotransferrin*". Such a human *lactoferrin* exists in milk of mammals including human milk, tears, saliva, mucosal secretions, and the secondary granules of polymorpho-nuclear leucocytes. It was first discovered by Peter Sorenson in 1939 and initially named "red protein" of human milk.

The *lactoferrin* (Lf) was isolated and purified from cow's milk at the first time. Since the first discovery, Lf has been isolated and purified in milk of other mammals such as human being, mouse, goat, rabbit, dog, etc. Human milk has a high content of the *lactoferrin*, for example, in the range of 6 to 8 mg/ml during the colostral phase. However, the *lactoferrin* content in human milk is decreased to about 2 mg/ml in the lactiferous phase. If the human milk is infected with bacteria during the lactiferous phase, the *lactoferrin* content in the infected milk is abruptly raised more than 30 times as high as the normal *lactoferrin* content.

Human *lactoferrin* (hLf) is a glycoprotein whose molecular weight is 78 kDa, composed of a single polypeptide chain containing 691 amino acids. Here, the single polypeptide chain is composed of a 2-fold internal repeating unit with two folded

globular lobes. That is, human *lactoferrin* (hLf) has C and N lobes constituting C and N terminals, respectively. The two lobes have the very similar structure with a high degree of homology (more than about 40%) between the C and N terminals. With the recent advance of X-ray chrystallography, the three-dimensional structure of *lactoferrin* has been determined such that each of the C and N terminals has a site to bind one iron with high affinity and that one *lactoferrin* molecule reversibly binds two ferric ions (Fe^{+3}) (Anderson et al., 1989). Such a *lactoferrin* is present in either iron-free (i.e., apo-type) or iron-saturated state (i.e., holo-type) depending on whether it binds irons, which in turn determines the biological properties of *lactoferrin*. The apo-type *lactoferrin* is present in normal human milk. All kinds of *lactoferrin* are almost stable under the acid condition in relation to *transferrin* and releases irons at a defined pH value.

Lactoferrin, which is one of non-immunoglobulin protective proteins secreted from exocrine glands, directly or indirectly participates in the anti-bacterial mechanism and thereby affects the anti-viral action. *Lactoferrin* possesses anti-bacterial activities against various microorganisms in the state of *in vitro* and *in vivo*. Especially, *lactoferrin* in the iron-free state (apo-type) has anti-microbial activities against gram-negative bacteria such as *E. coli, Kleabsiela pnumoni* and *Aerobacter aerogenes*, because it chelates with Fe^{3+} ions necessary for the microorganisms and thus inhibits the growth of the microorganisms. According to an *in vitro* experiment, it has been demonstrated that *lactoferrin* had a potent anti-bacterial activity like antibiotics against 99.99% of bacterium such as *Bacillus, E. coli* and *Salmonella* in one hour. *Lactoferrin* also participates in mechanisms involving an iron-binding action to inhibit the growth of microorganisms and abruptly deteriorate the bacteria viability. In the mechanisms, *lactoferrin* not only damages the outer membrane of gram-negative bacteria to release a large amount of lipopolysaccaride constituting the outer membrane and thereby destroy the permeability barrier of the membrane but also increases the sensitivity of the

microorganisms to hydrophobic antibiotics such as lysozyme or rifampicin, thus reducing the resistance of the microorganisms to antibiotics. In spite of the anti-bacterial action against pathogenic organisms, it is reported that *lactoferrin* has no anti-bacterial action against bacterium beneficial to human such as *Lactobacillus* or *Bifidus*. Thus *lactoferrin* contributes to protection of newborn babies against microbial infection. Furthermore, *lactoferrin* is also found in a small amount in blood and secreted from neutrophil. Such a *lactoferrin* is a principal constituent of the secondary granules of the neutrophil and is secreted in a large amount in an inflammatory response. There are some cases that the *lactoferrin* directly has a synergy effect with lysozyme or IgA in living bodies against infected pathogenic microorganisms. As the *lactoferrin* plays an important role in the protective mechanism against infectious hosts, patients who cannot produce *lactoferrin* in the body are seriously deteriorated in resistance to various diseases with an increase in the possibility of infection with bacteria or fungi. Besides, the *lactoferrin* serves as a mediator in cell proliferation or iron transport absorption.

BACKGROUND ART

Despite that *lactoferrin* has the various functions as previously described, many studies have not been made on the human *lactoferrin* because only a small amount of *lactoferrin* is contained in blood or other bodily fluids and there is a limitation in the available quantity of colostrums sample abundant in *lactoferrin*. As human milk is regarded as a great importance, careful studies on the *lactoferrin* are being made to use a genetic engineering approach to establish the base for industrial application of microorganisms in regard to cloning and expression of human *lactoferrin* (hLf) DNA in microorganisms. However, as described above, there is still a difficulty in using *E. coli* used widely in genetic engineering as an expression strain for human *lactoferrin* (hLf) unless a special recombinant plasmid for *E. coli* is produced.

It is also of great importance to verify the biological activity of the produced recombinant *lactoferrin*, since the normal bacteria or yeasts are mostly improper as a host due to the anti-bacterial activity of *lactoferrin* itself and, if used as a host, inadequate to industrial uses in such a small quantity available. To solve this problem, Ward et al. (1992) successfully produced the recombinant *lactoferrin* using fungi such as *Aspergillus indulans* or *oryzae*. But, there was a limitation in that the *lactoferrin* was expressed in such a small amount of 5 to 25 mg/l in relation to the *lactoferrin* expressed from an insect cell. Furthermore, because the recombinant *lactoferrin* produced from the fungi was analyzed for its biological activity only in terms of affinity with Fe^{58} labeled with radioactive isotopes, there was still remained a question as to whether the recombinant *lactoferrin* actually had an anti-bacterial action on the pathogenic microorganisms.

Meanwhile, studies concerning production of human *lactoferrin* using cell of the higher animals were attempted, but because of the high cost concerning the cultivation process, it is said that the application is difficult.

Thus, researchers in this art have shown some interest in producing human *lactoferrin* using insects cell which can be massively produced at a low cost. For example, it was reported that there were studies done in producing human *lactoferrin* using insects cell(Salmon et al., "Characterization of Human Lactoferrin Produced in the Baculovirus Expression System" in Protein Expr. Purif., vol. 9(2), 203-210, 1997). However, with such method, obtained human *lactoferrin* were very small amount of 15 mg/l. It was estimated that because of the sequence of human *lactoferrin* secretion did not well worked on insect cell. Also, 5% calf serum was used in such method, as a result, purification of human *lactoferrin* was bad.

To overcome the above problems, the present inventor has contrived a novel

method for mass-production of human *lactoferrin* in a simple way and a verification method for the biological activity of the recombinant human *lactoferrin*.

It is, therefore, an object of the present invention to provide a method for massively producing human *lactoferrin* using an insect cell.

It is another object of the present invention to provide a recombinant insect cell for producing a human *lactoferrin* protein.

It is still another object of the present invention to provide a biological verification method for a recombinant *lactoferrin* produced from an insect cell.

DISCLOSURE OF INVENTION

The present invention relates to a method for producing human *lactoferrin* comprising the steps of transducing a human lactoferrin gene into an insect cell by gene recombination, cloning and expressing human lactoferrin in the insect cell, and using the insect cell to produce the human lactoferrin. The present invention also relates to a

verification method for the biological activity of the recombinant human *lactoferrin.*

It is, therefore, an object of the present invention to provide a method for massively producing human *lactoferrin* using an insect cell.

It is another object of the present invention to provide a recombinant insect cell for producing a human *lactoferrin* protein.

It is still another object of the present invention to provide a biological verification method for a recombinant *lactoferrin* produced from an insect cell.

DISCLOSURE OF INVENTION

The present invention is related to a method for producing human *lactoferrin* comprising the steps of transducing a human lactoferrin gene into an insect cell by gene recombination, cloning and expressing human lactoferrin in the insect cell, and using the insect cell to produce the human lactoferrin. The present invention is also related to a verification method for the anti-bacterial activity of the recombinant human *lactoferrin.*

Reference will now be made to Figs. 1 and 2 as to a method for producing human *lactoferrin* using an insect cell according to the present invention.

The novel method for producing human *lactoferrin* by using an insect cell comprises the following steps: (a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to prepare a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK; (b) cotransfecting the recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to prepare a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from the recombinant insect cell; and (c) producing human *lactoferrin* from the recombinant insect cell Sf-Lf 6.

In the step of producing the recombinant insect cell, the culture medium in which the recombinant insect cell has been cultured is subjected by centrifugal separation to obtain a progeny virus originated from the insect cell.

To prepare the recombinant insect virus, the transfer vector 1 is first combined with the recombinant plasmid 2 to produce the recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK, and the recombinant expression vector is cotransfected with the help vector pBacPAK6 4 into the insect cell Sf9 5 in a culture medium to produce the recombinant insect cell Sf-Lf 6, from which the recombinant insect virus is produced.

The most widely used insect cell is *Spodoptera frugiperda* (Sf9) originated from army worm. The Sf9 cell line is also used as a host cell in the present invention. The Sf9 cell line, if infected with an insect virus such as *Baculovirus*, promotes synthesis of a mucous protein called polyhedrin. This means that the polyhedrin promoter for synthesizing polyhedrin in the Baculovirus is highly activated. It is stated in the related reports that the polyhedrin was produced in the insect cell in an amount of 1 to 500 mg/l and that the concentration of the external protein expressed depended on the type of protein or gene (Kaplan et al., 1990; Davidson et al, 1990; and Kaplan et al., 1991). The insect cell was highly analogous to the higher animal cells such as mammalian cells in terms of glycoprotein, phosphorylation, fatty acid acylation, amidation, and proteolytic processing, so that most higher animal cell proteins expressed in the insect cell had biological activities. The human *lactoferrin* as used herein was also verified to have a biological activity.

The expression vector used in the present invention is an insect virus DNA and the most widely used expression vector is multiple polyhedrosis virus AcMNPV called *Autographa califonica*. The life cycle and the infection cycle of the *Baculovirus* have been reported in the related document, King L.A and R.D. Possee, The Baculovirus

Expression System, A laboratory guide, HAPMAN and HALL. As stated above, the present invention uses an expression vector (pBacPAK, Clontech) originated from *Baculovirus*.

In the present invention, there are used various biochemical and molecular biological methods in order to verify the production of human *lactoferrin* from the insect cell. For example, PCR, Southern blot analysis and Western blot analysis are performed to support a fundamental study for producing a gene recombinant *lactoferrin* in the molecular level.

According to the present invention, the recombinant *lactoferrin* produced from the insect cell is biologically verified in a new way. That is, the recombinant *lactoferrin* is extracted from the insect cell and mixed with pathogenic bacteria such as *Pseudomonas cepacia, Pseudomonas putida, Pseudomonas fluorescence,* Salmonella *typhimurium* and *E. coli*, after which it is observed how much of the pathogenic bacteria have been destroyed in one hour at maximum.

On the other hand, the recombinant insect cell Sf-Lf used in the present invention is considered as an ideal system for mass production of human *lactoferrin* because it can be cultured in a flask due to its susceptibility of suspension culture and requires neither CO_2 unlike the higher animal cells nor fetal bovine serum (FBS) in cultivation.

BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a flow chart showing a process of producing a recombinant human *lactoferrin* using an insect cell according to the present invention.

Fig. 2 is a flow chart showing a process of producing a recombinant expression

vector pBacLf according to the present invention.

Fig. 3 is a photograph showing the electrophoresis pattern of the recombinant expression vector pBacLf after cleavage with a restriction enzyme according to the present invention.

Fig. 4 is a photograph of the agarose gel electrophoresis pattern showing that a human *lactoferrin* cDNA has been cloned from the recombinant baculovirus DNA isolated from the recombinant insect cell Sf-Lf according to the present invention.

Fig. 5 is a Southern blot photograph showing that a human *lactoferrin* DNA was cloned from the recombinant virus DNA obtained in Fig. 4.

Fig. 6 is a Western blot photograph and SDS-PAGE photograph showing that the recombinant insect cell Sf-Lf expresses and produces the human *lactoferrin* protein.

BEST MODE FOR CARRYING OUT THE INVENTION

EXAMPLE 1: Cultivation of Insect Cell and Production of Recombinant Expression Vector pBacLf

A *Spodoptera frugiperda* ovary cell Sf9 was used as an insect cell and cultured at a low temperature of 28 °C. The insect cell Sf9 was infected with an *autographa californica* nuclear polyhedrosis virus (AcMNPV) (pBacPAK 6) and cultured in a Grace's medium containing 10% FBS, lactalbumine, hydrolysate and antimycotic antibiotics. The insect cell was commercially available from Invitrogen Inc. (PO Box 2312,9704CH Groningen, Netherlands). The virus, AcMNPV (pBacPAK6) was provided by Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA).

To transfer a *lactoferrin* gene into a baculovirus gene, a 2.1 kb full gene including the start codon and the signal sequence of the *lactoferrin* was produced from the existing recombinant plasmid for cloning a transfer vector (pBacPAK8, Clontech Co.) including

the polyhedrin promoter site (5.5 kb), and then inserted into *E. coli* in the same direction of the polyhedrin promoter to prepare a recombinant expression vector. The transfer vector (pBacPAK8) was commercially available from Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA). The recombinant expression vector thus obtained was treated with a restriction enzyme to verify the *lactoferrin* gene, which was named "pBacLf". A process for producing the expression vector pBacLf is illustrated in Fig. 2.

Fig. 3 is a photograph showing the electrophoresis pattern of a selected recombinant expression vector pBacLf after cleavage with a restriction enzyme, wherein lane 1 is a size maker (λ/BstE I); lane 2 is the super coil of the recombinant expression vector pBacLf; lane 3 is the recombinant expression vector pBacLf cleaved with restriction enzymes BamH and Not I to verify the cleaved *lactoferrin* gene at 2.1 kb in terms of the full size of *lactoferrin*; lane 4 is the recombinant expression vector treated with a restriction enzyme, Eco R V to verify the *lactoferrin* gene; lane 5 is the recombinant expression vector treated with a restriction enzyme, Sma I to verify the *lactoferrin* gene; lane 6 is the recombinant expression vector treated with a restriction enzyme, Bgl II to verify the *lactoferrin* gene; lane 7 is the recombinant expression vector treated with a restriction enzyme, Pst I to verify the *lactoferrin* gene; and lane 8 is a size marker. As shown in Fig. 3, a human *interferrin* gene shows at 2.1 kb when the recombinant expression vector was cleaved with restriction enzymes BamH I and Not I into a fragment

EXAMPLE 2: Selection of a Recombinant Insect Cell Sf-Lf and Identification of a Recombinant Virus

Sf9 cells were inoculated in an amount of about 1.0×10^6 cells in a Grace's basic medium containing 10% FBS and were cultured for 4 hours. The insect cell was washed with the Grace's basic medium twice and remained at the ambient temperature for

30minutes. A mixture of a virus DNA (BacPAK 6, Clontech Co.) and a recombinant transfer vector prepared for liposome-mediated transfection, together with lipofectin was dropped on a cell monolayer. The mixture was added to the Grace's medium containing serum and antibiotics and was cultured at 28 °C for 5 days. The supertant was diluted with the culture solution in ten stages, 3 to 5 times and was inoculated into the insect cell Sf9 cultured in a monolayer on a 60mm-diameter plate. When the virus was adsorbed, the dissolved agarose-containing medium was hardened on the insect cell. After 6 to 7 days, the insect cell with the agarose-containing medium was dyed with neutral red, which dyes dead cells distinguished and forms a plaque. A microscope was used to select a plaque in which the polyhedrin by the infection with the recombinant virus was not formed. The plaque with the agarose was sucked up with a Pasteur pipette and was suspended in a 1ml medium. In order to verify whether the recombinant virus contains the *lactoferrin* gene, another insect cell Sf9 cultured in a new Grace's medium was infected with the recombinant virus and the recombinant virus DNA was isolated, after which the electrophoresis patterns of the isolated DNA were compared by agarose gel electrophoresis. By polymerase chain reaction (PCR) using a primer capable of amplifying *lactoferrin*, it was identified and after treatment with a restriction enzyme, the *lactoferrin* gene (2.1 kb) was identified by Southern blot analysis.

Fig. 4 shows that the *lactoferrin* cDNA (2.1 kb) was cloned from the recombinant virus DNA isolated from the recombinant insect cell Sf-Lf, in which lane 1 shows a size marker (λ/BstE II); lane 2 is a negative control (pBacPAK8); and each of lane 3 and 4 is a recombinant virus DNA. It is seen from lane 3 that there has been amplified the human *lactoferrin* cDNA (2.1 kb) using a primer exclusively amplifying the human *lactoferrin* from the recombinant virus DNA.

Fig. 5 verifies the *lactoferrin* DNA (2.1 kb) by Southern blot analysis for the recombinant virus DNA treated with restriction enzymes such as BamH I, Not

I and Acc I, based on the fact verified in Fig. 4, in which lane 1 shows a recombinant virus intact DNA; lane 2 is a recombinant virus DNA (treated with BamH I/Not I); lane 3 is a recombinant virus DNA (treated with Acc I); lane 4 is a recombinant virus DNA (treated with BamH I/Not I/Bgl II); lane 5 is an amplified PCR *lactoferrin* gene produced from the virus DNA; lane 6 is a DIG-labeled size marker; lane 7 is a super coil DNA of the recombinant plasmid (pBacPAK8); and lane 8 is a negative control (pGEMLf) (treated with BamH I/Not I).

The negative control was pBacPAK8 and the positive control was lactoferrin-gene-containing pGEMLf cleaved with a restriction enzyme BamH I/Not I. The probe as used herein was a part of the N-lobe of DIG-labeled *lactoferrin* cDNA. As shown in Fig. 5, the band was observed at the same location (2.1 kb) of the positive control in the recombinant baculovirus DNA. It demonstrates that the selected recombinant virus of the present invention contained human *lactoferrin* DNA.

EXAMPLE 3: Expression of Human *Lactoferrin* from Recombinant Insect Cell (Sf-Lf) and its Verification

To verify expression of protein, the recombinant cell was pulverized with a cell lysis buffer (50 mM Tris-HCl, pH 8.0, 5% 2-mercaptoethanol, 0.4% w/v SDS, 10 mM EDTA) and applied to coomassie-blue polyacrylamide gel running, after which a Western blot analysis was performed using anti-*lactoferrin* (anti-Lf).

As shown in Fig. 6, the recombinant viral stock was inoculated into the insect cell Sf9 and the cells were pulverized with the insect host cell Sf9 at the fourth day. The supernatant was collected for a Western blot analysis with anti-*lactoferrin* (anti-Lf) Ab and SDS-PAGE and, as a result of which the band was color-developed at the same location (80 kDa) of colostrums used as a positive control and purified *lactoferrin* protein. This demonstrates that the human *lactoferrin* protein was produced in the recombinant

insect cell (Sf-Lf). Lane 1 shows a protein size marker, lane 2 is a colostral soup, lane 3 is the insect cell Sf9, lanes 4 and 5 are the recombinant insect cells. A densitometry revealed that the expressed *lactoferrin* was in an amount of more than 800 mg/l. The amount expressed is much higher than the amount of *lactoferrin* expressed from *Aspergillus Nidulans* or *Aspergillus Oryzae* and shows economically high productivity.

EXAMPLE 4: Verification of Anti-bacterial Activity of Recombinant *Lactoferrin*

To measure the anti-bacterial activity of the recombinant insect cell (Sf-Lf) against pathogenic microorganisms, the insect cell (Sf-Lf) was pulverized by a freeze and thaw method and the supernatant was mixed with the pathogenic microorganisms in the *lactoferrin* concentration of about 250 μg/ml. The mixture was then smeared to a plate count agar plate at intervals of 0, 15, 30, 45 and 60 minutes.

It can be seen from the cell count of Table 1 that the *lactoferrin*-containing the supertant alone destroys the pathogenic microorganisms within one hour. For the negative control, insect cell (Sf) was pulverized and subjected to microassay on pathogenic microorganisms in the same way as for the recombinant insect cell (Sf-Lf), in which case there was no decrease in the number of cells.

The above results revealed that the *lactoferrin* protein produced from the recombinant insect cell (Sf-Lf) possessed an anti-bacterial activity.

INDUSTRIAL APPLICABILTY

The recombinant insect cell (Sf-Lf) produced by the method for producing human *lactoferrin* using an insect cell according to the present invention is advantageous in that it can be cultured in a flask due to its susceptibility of suspension culture, requiring neither carbon dioxide (CO_2) unlike the higher animal cells, nor fetal bovine serum (FBS) in the culture, as a result of which using the recombinant insect cell allows mass production of human *lactoferrin* at a low cost in a simple way.

Table 1

Strain	Cell Count (cfu/ml)									
	*E. coli 300		*S. typhimurium 114		@P. putida		@P. fluorescence		@P. cepasia 9613	
	-	+	-	+	-	+	-	+	-	+
0 min	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	6.7×10^6	$>10^7$	1.8×10^6	5×10^3	1.4×10^3
15 min	$>10^7$	$>10^5$	$>10^7$	5.0×10^4	$>10^7$	3.0×10^4	$>10^7$	1.4×10^4	5×10^3	9.8×10^2
30 min	$>10^7$	1.3×10^4	$>10^7$	1.2×10^4	$>10^7$	6.9×10^3	$>10^7$	4.0×10^3	5×10^3	2.1×10^2
45 min	$>10^7$	6.2×10^3	$>10^7$	2.0×10^3	$>10^7$	3.0×10^3	$>10^7$	2.9×10^3	5×10^3	10
60 min	$>10^7$	10^2	$>10^7$	1.2×10^2	$>10^7$	10^2	$>10^7$	5×10^2	5×10^3	0

Note: * indicates an animal pathogenic bacterium; @ indicates a food contaminant bacterium; + indicates a culture with *lactoferrin*; and – indicates a culture without *lactoferrin*

CLAIMS

1. A method for producing human *lactoferrin* by using an insect cell comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from said recombinant insect cell; and

(c) producing human *lactoferrin* from said recombinant insect cell Sf-Lf 6.

2. The method of claim 1, wherein said producing a recombinant insect virus step further comprises the step of performing a centrifugal separation of the culture medium containing the recombinant insect cell cultured in the producing step (b) to obtain a progeny virus from the insect cell contained in the upper layer.

3. A human *lactoferrin* produced by a method comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from said recombinant insect cell; and

(c) producing human *lactoferrin* from said recombinant insect cell Sf-Lf.

4. A recombinant insect virus produced by a method comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce and culture a recombinant insect cell Sf-Lf 6; and

(c) producing a recombinant insect virus from said recombinant insect cell Sf-Lf.

5. A biological verification method for a recombinant human *lactoferrin,* comprising the steps of:

mixing human *lactoferrin* produced by the method of claim 1 with a pathogenic microorganism; and,

measuring anti-bacterial activity of said mixture against the pathogenic microorganism.

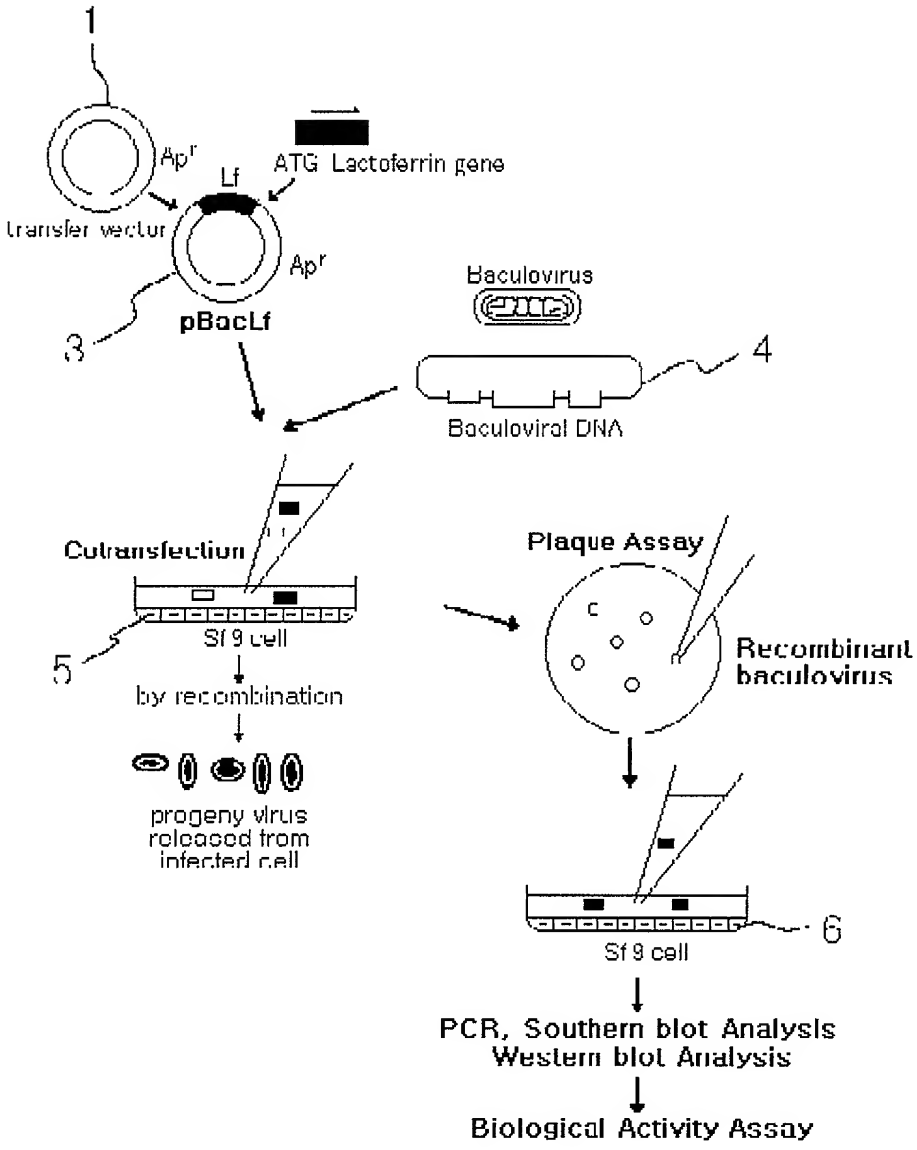
6. The method of claim 5, wherein said pathogenic microorganism is selected from the group consisting of *Pseudomonas cepacia, Pseudomonas putida, Salmonella typhimurium*, *Pseudomonas fluorescence* and *E. coli.*

ABSTRACT

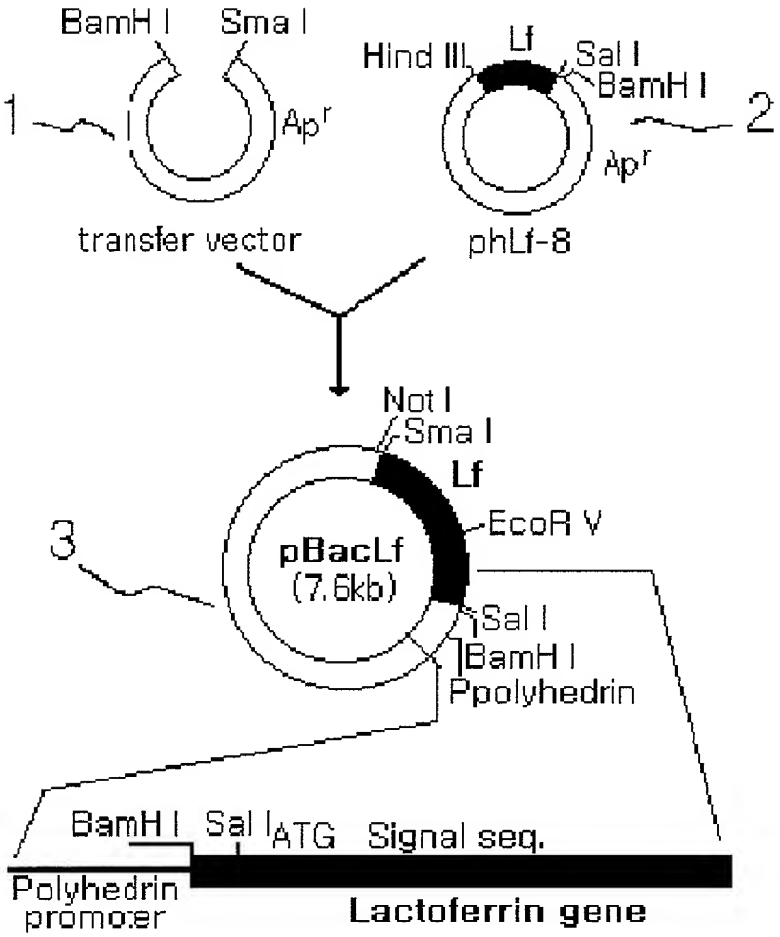
The producing process comprises Lactoferrin gene's producing recombined expression vector(pBacLf)(3) which is adjusted polyhedrin promoter in vector(pBacPAK) by combining transfer vector and remixed plasmid ; producing recombined insect cell(Sf-Lf)(6) by cotransfection of the recombined revealed vector with help vector(pBacPAK6) at insect-cell in a culture medium ; producing recombined insect virus from the recombined insect cell ; and producing human Lactoferrin from the recombined insect-cell.

In the invention, biological verifying method of recombined human Lactoferrin is measuring extinct rate of disease-causing germs after extracting recombined human Lactoferrin from recombined insect-cell and mixing it with disease-causing bacteria like *Pseudomonas cepacia, Pseudomonas putida, Pseudomonas fluorescence, Salmonella typhimurium, E. coli.*

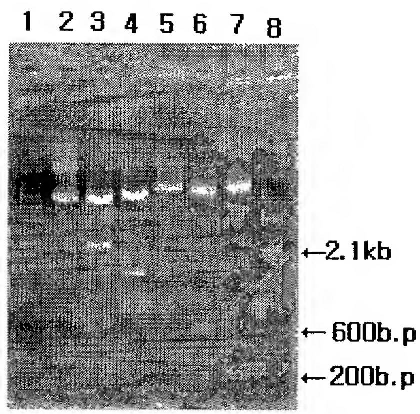
【Fig. 1】


1
Ap^r
Lf
ATG Lactoferrin gene
transfer vector
Ap^r
pBacLf
3
Baculovirus
4
Baculoviral DNA
Cotransfection
Sf 9 cell
5
by recombination
progeny virus
released from
infected cell
Plaque Assay
Recombinant
baculovirus
Sf 9 cell
6
PCR, Southern blot Analysis
Western blot Analysis
Biological Activity Assay

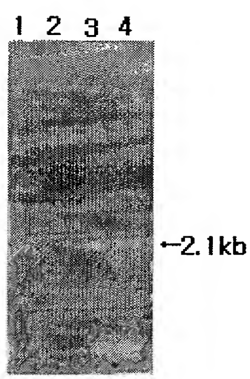
【Fig. 2】



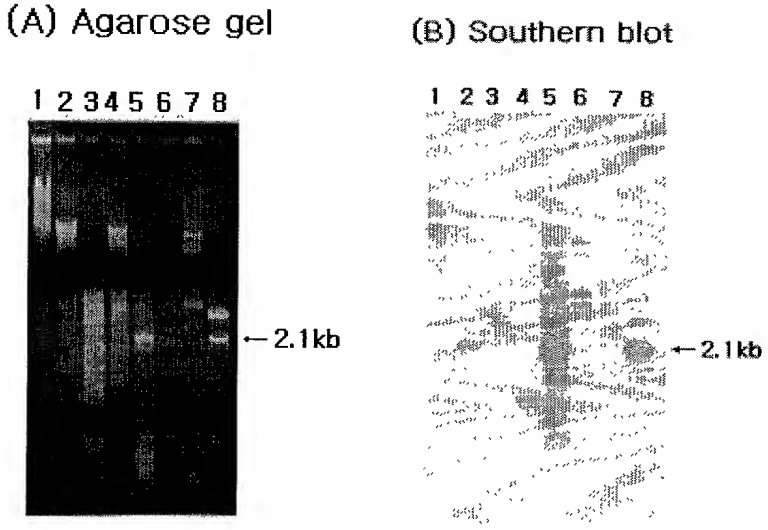
【Fig. 3】



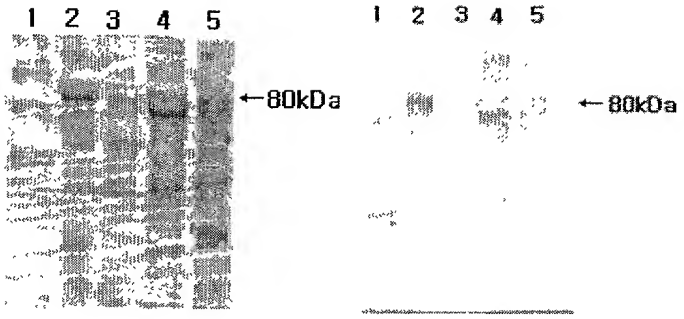
【Fig. 4】



【Fig. 5】



【Fig. 6】



DECLARATION

UTILITY PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is HUMAN LACTOFERRIN PRODUCED BY USING AN INSECT CELL AND METHOD USING THE SAME, the specification of which

CHECK ONE

[X] is attached hereto.

[ ] was filed on _____ as
Application Serial No. _____
and was amended on _____
(if applicable)

I have read the applicable statutes and rules reprinted on the reverse side of this declaration which I understand to describe subject matter which is material under 37 C.F.R. § 1.56(a).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Application Number	Country	Date of Filing	Priority Claimed	
10-1999-30517	Republic of Korea	July 27, 1999	Yes ☑	No ☐

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Number	Date of Filing	Status
PCT/KR00/00810	July 26, 2000	Pending

DECLARATION, continued...

APPLICABLE STATUTES & RULES

37 C.F.R. § 1.56 - DUTY OF DISCLOSURE; FRAUD; STRIKING OR REJECTION OF APPLICATIONS

(a) A duty of candor and good faith toward the Patent and Trademark Office rests on the inventor, on each attorney or agent who prepares or prosecutes the application and on every other individual who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application. All such individuals have a duty to disclose to the Office information they are aware of which is material to the examination of the application. Such information is material where there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent. The duty is commensurate with the degree of involvement in the preparation or prosecution of the application.

Information relating to the following factual situations enumerated in 35 U.S.C. § 102 and § 103 should be considered material under 37 C.F.R. § 1.56(a):

A person shall be entitled to a patent unless --

(a) The invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent, or

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States, or

(c) he has abandoned the invention, or

(d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months before the filing of the application in the United States, or

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or

(f) he did not himself invent the subject matter sought to be patented, or

(g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other.

35 U.S.C. § 103 - CONDITIONS FOR PATENTABILITY; NON-OBVIOUS SUBJECT MATTER

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

35 U.S.C. § 119 - BENEFIT OF EARLIER FILING DATE IN FOREIGN COUNTRY; RIGHT OF PRIORITY (Applicable Portion)

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which has been patented or described in a printed publication in any country more than one year before the date of the actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

35 U.S.C. § 120 - BENEFIT OF EARLIER FILING DATE IN THE UNITED STATES

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, by the same invention shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or on an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

35 U.S.C. § 112 - SPECIFICATION (Applicable Portion)

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

DECLARATION, continued...

201	FULL NAME OF INVENTOR:	Hyune Hwan LEE			
	RESIDENCE:	City Seoul	State or Foreign Country Republic of Korea		Country of Citizenship Republic of Korea
	POST OFFICE ADDRESS:	9-802, Samick APT, Myungil-Dong Kangdong-Gu			
		City Seoul	State or Province	Zip Code	Country Republic of Korea

202	FULL NAME OF INVENTOR:	Yun-Hee CHANG			
	RESIDENCE:	City Inchon-Si	State or Foreign Country Republic of Korea		Country of Citizenship Republic of Korea
	POST OFFICE ADDRESS:	978-13(34/2), Gyesan-1Dong			
		City Inchon-Si	State or Province Gyeyang-Gu	Zip Code 407-051	Country Republic of Korea

203	FULL NAME OF INVENTOR:	Chang-Hee KWEON			
	RESIDENCE:	City Kunpo-Si	State or Foreign Country Kyunggi-Do		Country of Citizenship Republic of Korea
	POST OFFICE ADDRESS.	A-202, Jangmi APT, 706-17, Kunyung-Dong,			
		City Kunpo-Si	State or Province Kyunggi-Do	Zip Code 435-050	Country Republic of Korea

204	FULL NAME OF INVENTOR:	FIRST Name	Middle Initial(s)	LAST Name	
	RESIDENCE:	City	State or Foreign Country		Country of Citizenship
	POST OFFICE ADDRESS.				
		City	State or Province	Zip Code	Country

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

SIGNATURE of Inventor 201 Hyune Hwan LEE Hyune Hwan Lee	SIGNATURE of Inventor 203 Kweon, Chang-Hee Chang-Hee KWEON
Date 24 January, 2002	Date 24 January, 2002
SIGNATURE of Inventor 202 Yun-Hee CHANG Chang, Yun-Hee	SIGNATURE of Inventor 204
Date 24 January, 2002	Date

(Signatures should conform to names as presented at 201 *et seq* above.)

POWER OF ATTORNEY

We/I, Hyune Hwan LEE, Yun-Hee CHANG, & Chang-Hee KWEON, executed on the date indicated below do(es) hereby appoint as attorneys of record with full power of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: John K. Park, Reg. No. 37,904.

Send Correspondence to: JOHN K. PARK Park & Sutton LLP 3255 Wilshire Blvd., Suite 1110 Los Angeles, California 90010	Direct Telephone Calls to: 213-389-3777 Fax to: 213-389-3377

We/I, the undersigned, declare that we/I are(am) the (an) owner(s) of the above-mentioned application or, if the owner is a corporation, partnership, or other association, we/I are(am) authorized to make this appointment on behalf of the owner, and we/I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

201	FULL NAME OF INVENTOR:	Hyune Hwan LEE		
	POST OFFICE ADDRESS:	9-302, Samick APT, Myungil-Dong Kangdong-Gu		
	City, State or Province: Seoul	Zip Code:	Country: Republic of Korea	
	Date: 24 Jan, 2002	SIGNATURE: Hyune Hwan Lee		

202	FULL NAME OF INVENTOR:	Yun-Hee CHANG		
	POST OFFICE ADDRESS:	978-13(34/2), Gyesan-1Dong, Gyeyang-Gu		
	City, State or Province: Inchon-Si	Zip Code: 407-051	Country: Republic of Korea	
	Date: 24 Jan, 2002	SIGNATURE: Chang, Yun-Hee		

203	FULL NAME OF INVENTOR:	Chang-Hee KWEON		
	POST OFFICE ADDRESS:	A-202, Jangmi APT, 706-17, Kumjung-Dong		
	City, State or Province: Kunpo-Si, Kyunggi-Do	Zip Code: 435-050	Country: Republic of Korea	
	Date: 24 Jan, 2002	SIGNATURE: Kweon, Chang-Hee		

204	FULL NAME OF INVENTOR:	FIRST Name	Middle Initial(s)	LAST Name
	POST OFFICE ADDRESS:			
	City, State or Province		Zip Code	Country
	Date	SIGNATURE		